

AD _____

Award Number: DAMD17-99-1-9394

TITLE: Involvement of a Novel Rho GTPase Activating Protein in
Breast Tumorigenesis

PRINCIPAL INVESTIGATOR: Rajendra P. Kandpal, Ph.D.

CONTRACTING ORGANIZATION: Fordham University
Bronx, NY 10458

REPORT DATE: August 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040226 027

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY
(Leave blank)

2. REPORT DATE

August 2003

3. REPORT TYPE AND DATES COVERED

Final (1 Jul 1999 - 31 Jul 2003)

4. TITLE AND SUBTITLE

Involvement of a Novel Rho GTPase Activating Protein in Breast Tumorigenesis

5. FUNDING NUMBERS

DAMD17-99-1-9394

6. AUTHOR(S)

Rajendra P. Kandpal, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Fordham University
Bronx, NY 10458

E-Mail: kandpal@fordham.edu

8. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING / MONITORING

AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

We had previously shown that expression of a novel transcript that had sequence similarity to rat Rho GAP sequence was altered in breast carcinoma cell lines. We believed that the putative Rho GAP is functionally active. Rho family of proteins shares homology with the Ras super family. Our working hypothesis was that breast carcinoma where Ras mutations have not been detected could still arise from aberrant Ras signaling by virtue of loss of activity of members of Rho family or factors/proteins that affect the activity of Rho proteins. We have bacterially expressed the putative Rho GAP and shown it to have specificity toward RhoA. To further associate the biochemical activity with its well-established physiological role we have demonstrated that transfected Rho GAP can alter actin organization of fibroblast cell lines. We have transfected breast cancer cells with RhoGAP expression construct and shown it to suppress cell growth. This growth suppression pathway is mediated via p21. To better characterize the physiological significance of RhoGAP we have used the yeast two-hybrid system to isolate interacting proteins. The protein was shown to interact with alpha tubulin, TAX1 binding protein, SWI/SNF and HMG CoA reductase. These interactions provide a mechanism for statin induced inhibition of breast carcinoma cells and indicate as yet uncharacterized involvement of RhoGAP in other processes.

14. SUBJECT TERMS

Breast Cancer

15. NUMBER OF PAGES

24

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT

Unclassified

18. SECURITY CLASSIFICATION
OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION
OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	
SF 298.....	1
Table of Contents.....	2
Introduction.....	3
Body.....	5
Key Research Accomplishments.....	9
Reportable Outcomes.....	10
Conclusions.....	11
References.....	12
Appendices.....	14

INTRODUCTION

The development of multicellular organisms is mediated by a highly complex and coordinated signaling network. The aberrant expression and/or function of various members of signaling pathways has been conclusively linked to developmental abnormalities and a variety of chronic diseases including cancer. This proposal addresses downstream elements of Ras signal transduction pathway that may mediate some aspects of malignant transformation. Ras- and Ras-related families, Rab, Rac and Rho are small GTP binding proteins. These proteins are key components of signal transduction pathways that link extracellular proliferation and/or differentiation signals such as growth factors and oncogenes to nuclear transcription of specific genes which promote these processes (1-3). Indeed, mutations affecting Ras and/or Ras pathway are frequently seen in human cancers including lung, colon, endometrium, ovary, pancreas, thyroid and a smaller proportion of breast cancers (4). It has been demonstrated in several cancers where Ras is not mutated that downstream effectors of Ras signaling pathway are involved in tumorigenesis. Ours is the first report implicating Rho GAP, a downstream member of the Ras-Rho signaling cascade, in breast tumorigenesis.

The Rho family of small GTP-binding proteins which include Rho, Rac, and cdc42 belong to the Ras superfamily (2), and comprises several known proteins (Rho A, Rho B, Rho C, Rho D, Rho E/Rnd3, Rnd1/Rho6, Rnd2/Rho7, Rho G, Rac 1, Rac 2, Rac 3, Cdc42, and TC10). These proteins bind GTP and hydrolyze it via low levels of intrinsic GTPase activity. They function as molecular switches in the transduction of signal generated by the activation of a variety of cell surface receptors including the EGF receptor, the erbB-2 gene product, the CSF-1 receptor, and the cell adhesion receptor integrins (2,3). When cells receive an appropriate stimulus, these GTPases are converted to the active GTP-bound state by activated guanine nucleotide exchange factors (GEFs), whereas the GTP-bound form is later rendered inactive by the GTPase-activating proteins (GAPs) (5). The activated Rho GTPases interact with multiple downstream effector targets which serve to transmit the incoming signals from the small G-proteins leading to cellular responses such as actin-cytoskeleton changes (3), cell polarization (2), transcriptional activation (6-8), focal adhesion formation (9), and stimulation of DNA synthesis (10). Rho is required for growth factor induced formation of stress fibers and focal adhesions (11), regulation of cell morphology (12), cell aggregation (13), cell motility (14), and cytokinesis (15).

The activity of Rho is negatively regulated by GTPase activating proteins (GAPs). Several Rho GAPs have been identified, and some of the GAPs have been shown to stimulate specific Rho family proteins preferentially (16). Some well characterized GAPs include Rho GAP, Bcr, and a specific modulator of Rho i.e. p190 (11). Expression of GAP domain of p190 in Swiss 3T3 cells has been shown to cause changes in morphology and lysophosphatidic acid (LPA)- and serum- induced stress fiber formation (11). An aberrant expression, regulation and/or activity of the proteins involved in cytoskeletal organization may lead to transformation of a normal cell, and alter the invasiveness and metastatic potential of a malignant cell.

The biochemical elements involved in regulation of Ras and Ras-related pathways are central to cancer biology and the normal processes of cell proliferation, differentiation and development. The activation of Rho by oncogenic Ras may affect cell-cell interactions and invasiveness that are characteristic of malignant cells (17,18). Recent studies have provided direct evidence that indicate activated forms of Rho and Rac as oncogene products capable of mediating cell transformation and tumor formation (19). While Rho, Rac and cdc42 all seem to be essential for Ras transformation, Rho and Rac act in the Ras proliferation pathway (20,21), and cdc42 controls the Ras-mediated anchorage dependent growth property (22). The oncogenes that encode GEFs for specific Rho proteins (16) have been shown to stimulate GDP/GTP exchange of the GTPases, resulting in various transforming phenotypes in many

cell types, including epithelial and fibroblast cells. The Rho proteins have also been implicated in integrin-mediated cell response to extracellular matrix (23). The expression of constitutively active Rac or cdc42 in breast epithelial cells has been shown to disrupt the formation of differentiated, polarized epithelium in three dimensional collagen gels and induce integrin-mediated motility and invasion through collagen matrices, thus implicating Rho GTPases in mammary cell tumorigenesis (24).

The molecular links to the diverse biological activities of Rho proteins are based on the putative effectors that have been identified so far. The candidate targets for these proteins were identified by their specific binding strengths to the active Rho GTPases. For example, the p85 regulatory subunit of PI3 kinase, which interacts with cdc42 and Rac both in vivo and in vitro may be involved in cell motility (24-26). Likewise, several novel serine/threonine kinases or p21-activated kinases (PAK) were discovered by using an overlay affinity assay (27). Data base searches with these proteins as query sequences identified a large family of proteins sharing a conserved cdc42/Rac interactive binding (CRIB) motif of PAK (28), many of which were shown to be candidate effectors for cdc42 or Rac. Affinity chromatography led to the identification of several other novel targets such as Rhophilin, protein kinase N (PKN), IQGAPs, N-WASP, p140mDia, Rho kinase (ROK), p120 Ack, N-WASP, and Por1(2), which are involved in a wide spectrum of Rho-mediated cellular responses. Several lines of evidence have implicated specific Rho proteins as key downstream mediators of Ras transformation, and indicated clearly that Ras must utilize effectors other than Raf. One of the important observations suggesting such a link was identification of a p120 GAP-associated protein, p190, as a GAP for RhoA and to some degree cdc42 and Rac (29). The pathways downstream of Rho protein effectors that cause cytoskeletal changes and other nuclear events remain mostly unknown. It warrants mention that the proposed experiments in this application to isolate Rho GAP interacting proteins (specific aim 3) assume significance, as these experiments may allow identification of molecular links between signaling pathways.

The best characterized function of Rho family proteins involves their regulation of specific filamentous F-actin organization. The three major cytoskeletal protein networks, namely, actin filaments, microtubules, and intermediate filaments are involved in a variety of processes. Specifically, actin cytoskeleton is a highly dynamic structure responsive to diverse extracellular signals. Lamellopodia, membrane ruffles, actin stress fibers, focal adhesions, and filopodia are representative distinct actin-based structures, which are regulated by specific Rho family proteins in fibroblasts and other cell types (30). Because an altered morphological phenotype is a key feature of transformed cells, the Rho mediated pathways that control cytoskeleton reorganization may contribute to the general phenomena of transformation and invasiveness. The ability of a eukaryotic cell to maintain or change its shape and its degree of attachment to substratum in response to extracellular signals is largely dependent on the rearrangement of the actin cytoskeleton. Cytoskeletal rearrangements play a crucial role in processes such as cell motility, cytokinesis, cell growth, and contact inhibition- and alteration in these processes is a hallmark of tumor cells. The recognition in our laboratory that a novel Rho GAP expression is altered in breast tumors unrelated to BRCA1 and BRCA2 opens newer avenues to investigate this underexplored Rho GAP of the Rho signaling pathway and its potential involvement in breast tumorigenesis.

Body:

We had originally proposed to characterize a human Rho GTPase Activating Protein (GAP) that we had cloned from human chromosome 13q21. The specific aims proposed in the original application are stated below.

Specific aim 1: a) investigate the expression profile and level of Rho GAP expression in breast cancer cell lines and correlate lack of its activity to gene mutation, and b) express the novel Rho GAP, and determine its biochemical and functional properties such as GAP activity, substrate specificity and phospholipase C-delta stimulation.

Specific aim 2: Investigate the role of Rho GAP in normal breast cells using antisense constructs to sequester the native Rho GAP message and determine its effect on lysophosphatidic acid induced Rho-mediated actin reorganization and phenotypic changes in normal and cancer breast cells.

Specific aim 3: Use the yeast two hybrid system to identify other proteins that interact with the novel Rho GAP and are involved in Rho-mediated signaling.

Statement of Work:

The specific tasks for the entire grant period are described below.

Technical objective 1: a) Investigate the expression profile and level of active Rho GAP expression in breast cancer cell lines and correlate lack of its activity to gene mutation, and b) Express the novel Rho GAP, and determine its biochemical and functional properties such as GAP activity, substrate specificity, and phospholipase C- delta1 stimulation.

- | | |
|------------------------|--|
| Task 1: Months 1-2: | Verification of the gene sequence and its further characterization. |
| Task 2: Months 3-5: | Rho GAP expression profiles in various breast carcinoma cell lines. |
| Task 3: Months 6-9: | Correlation of GAP activity by nitrocellulose assay with SSCP gene Mutation. |
| Task 4: Months 10-12 : | Purification of proteins Rho GAP, Rho A and phospholipase C-d. |
| Task 5: Months 13-16: | Determination of enzymatic activity and substrate specificity. |

Technical objective 2: Investigate the role of Rho GAP in normal breast cells using antisense constructs to sequester the native Rho GAP message and determine its effect on lysophosphatidic acid (LPA) induced Rho-mediated actin reorganization, and phenotypic changes in normal and cancer breast cells

- | | |
|-----------------------|--|
| Task 6: Months 17-19: | Cloning of Rho GAP and its mutants in sense and antisense orientation. |
| Task 7: Months 20-23: | Cytoskeleton reorganization in transfected cells. |
| Task 8: Months 24-27: | Phenotypic changes in normal and breast carcinoma cell lines after transfection. |

Technical objective 3: Use the yeast two hybrid system to identify other proteins that interact with the novel Rho GAP and are involved in Rho-mediated signaling.

- | | |
|------------------------|---|
| Task 9: Months 28: | Cloning of Rho GAP in pBD-GAL4 cam vector. |
| Task 10: Months 29-32: | Isolation of interacting clones. |
| Task 11: Months 33-36: | Characterization of interacting clones by sequencing. |

The following section describes the progress made in specific tasks assigned for the grant.

Task 1:

The entire coding sequence and some 5' and 3' untranslated regions were present in the 4.8 Kb sequence determined for the RhoGAP sequence. The sequence has been deposited in the NCBI database with accession number AY366448. The specific features in the sequence are as follows.

- i) A START domain at the N-terminal region. The START domain, known to confer lipid binding ability, was first characterized in steroidogenic acute regulatory protein, homeodomain proteins of the plant *Glabra2* family, and bovine phosphatidyl choline-transfer protein (PC-T) (Ponting and Aravind, 1999; Christenson and Strauss, 2000). It can be speculated that START domain likely facilitates RhoGAP recruitment to the membrane where it can exert its biochemical and biological activity on distinct targets.
- ii) A conserved GAP domain found in RhoGAPs.
- iii) The protein has another motif- sterile alpha motif (SAM) that likely mediates protein interactions (Thanos et al., 1999).
- iv) The cDNA has several ATTTA motifs known as instability elements. These elements have been implicated in destabilizing the mRNA Caput et al., 1986; Shaw and Kamen, 1986) for several oncogenes and other transcripts involved in cell proliferation. The presence of these elements indicates that the RhoGAP message is likely under a stringent regulatory control.

Figure 1 shows alignment of GAP domain with other proteins.

Task 2-5:

The RhoGAP cDNA was used as a probe in a Northern blot containing total RNA from various breast cancer cell lines. We observed qualitative as well as quantitative changes. In general, the levels of transcript were relatively low in breast cancer cell lines as compared to normal breast cell line. Furthermore, MCF7 and MDAMB231 cells showed an altered size transcript. The results are shown in figure 2.

We attempted to use nitrocellulose overlay assay to profile the various targets of RhoGAPs in a variety of cell lines. However, these experiments were unsuccessful. We observed a large background that made detection of specific targets difficult. The SSCP analysis of various exons in cell lines revealed mobility changes in some cell lines indicating the alterations in sequences. The exact nature of these changes was not confirmed by sequencing.

The entire coding sequence of Rho GAP was cloned in a bacterial expression vector and the expressed as a GST fusion protein. RhoA, Rac1 and cdc42 expression constructs were obtained from Dr. A. Hall. The purified RhoGAP and Rho proteins were used to determine biochemical activity and specificity. RhoGAP was capable of stimulating the GTPase activity of RhoA, Rac1 and cdc42. However, it showed specificity toward GTPase activity of RhoA.

Determination of GAP activity of recombinant Rho GAP: The activity of purified Rho GAP was checked on as follows. The GTPase assay was performed according to the method of Hall and colleague. Fifty ng each of Rho A protein was preloaded with [γ - 32 P] GTP (6000 Ci/mmol, 10 μ Ci/ μ l) in 20 μ l of 20 mM Tris-HCl, pH 7.6, 0.1 mM DTT, 25 mM NaI, and 4 mM EDTA for 10 min at 30°C. The mixture was placed on ice and MgCl₂ added to a final concentration of 17 mM. Three μ l of the preloaded protein was diluted with buffer (20 mM Tris-HCl, pH 7.6, 0.1 mM DTT, 1 mM GTP, 1 mg/ml Bovine Serum albumin) to give a final volume of 30 μ l. A 5 μ l sample was removed (time zero) and diluted into 1 ml cold assay buffer (50 mM Tris HCl, pH 7.6, 50 mM NaCl and 5 mM MgCl₂). For

GAP stimulated GTPase assays, an aliquot of GAP proteins was added at this stage. The remainder of the reaction was incubated at 20°. Samples (5 µl) were removed at 5, 10, 15 min. intervals and diluted with cold assay buffer (1 ml). The reaction mixture was filtered through pre wetted nitrocellulose filters, and the amount of radioactivity remaining bound to the protein was determined by scintillation counting. The result was expressed in terms of % bound GTP remaining vs time. As shown in Table 1, the RhoGAP enhanced the GTPase activity of RhoA.

Tasks 6-8:

The full-length RhoGAP was cloned in pCDNA3.1 vector, and its ability to influence growth characteristics of breast cancer cells was evaluated in the following manner.

- i) MCF7 cells were transfected with either an empty vector or the RhoGAP construct.
- ii) MCF7 cells were transfected with RhoGAP followed by p21.
- iii) MDAMB231 cells were transfected with p21.
- iv) MDAMB231 cells were transfected with p21 followed by RhoGAP.

The transfected cells when compared with mock transfectants showed growth inhibition. Whenever the cells were doubly transfected there was no additive effect on growth inhibition. The double transfections were carried out as follows. The cells were transfected with one construct and allowed to grow for 8-12 hours before the second transfection was carried out. These experiments indicate that RhoGAP effects are mediated via p21. The data are shown in Figures 3 & 4.

Effect of Rho GAP expression on cell morphology: The best characterized effect of Rho proteins is on actin reorganization and cytoskeleton. We have standardized RhoGAP effects on NIH3T3 cell lines, a system best suited for investigating actin cytoskeleton. Briefly, NIH3T3 cells were grown in appropriate tissue culture medium and serum starved for a few hours. The cells were stimulated with growth factors and the cytoskeleton visualized by staining.

The Rho GAP-transfected and untransfected NIH3T3 cells were grown in appropriate culture medium containing 0.11 g/L sodium pyruvate 4.5g/L glucose, 10% fetal calf serum at 37°C in a CO₂ incubator. For analysis of growth factor induced actin reorganization, quiescent cells were seeded on glass coverslips. Serum was added to starved cells and incubated at 37° for 10 minutes. Following stimulation cells were washed with PBS containing 0.5 mM CaCl₂ and fixed for 20 minutes in 3% paraformaldehyde, washed to remove excess fixative, and then permeabilized in 0.2% Triton X-100. The coverslips were washed twice with PBS and then incubated with 200 µl of 0.1 µg/ml phalloidin for 30-40 minutes in the dark to localize actin filaments. As can be seen in figure 5 that growth factors induced actin reorganization was reversed in the presence of Rho GAP constructs. These studies demonstrate that the RhoGAP indeed influences actin reorganization and confirms the biochemical activity as established above with its physiological function.

Tasks 9-11:

We have used the Matchmaker two-hybrid kit (CLONTECH) to isolate cDNAs for proteins that interact with RhoGAP as per the manufacturer's protocol. The Rho GAP coding sequence was cloned in frame with the GAL4 binding domain in pGBKT7 vector. A human brain library prepared in the vector containing the GAL4 activation domain was used for isolating RhoGAP interacting clones. The interacting clones were selected by plating the transformed yeast cells onto a quadruple drop-out medium. The surviving colonies were transferred and grown onto a nylon membrane filter. The colonies were tested in a β-galactosidase assay by incubating the filter with X-GAL. The surviving

colonies were picked up, grown, and processed for DNA preparation. The DNA was used to transform *E.coli* to isolate plasmid DNAs. The clones were analyzed by sequence determination. The interacting clones were also confirmed in mammalian cells as described below (Table 2).

Several cDNAs were isolated in the preliminary screening. However, only a few passed the confirmatory test, and four of these clones were shown to interact in mammalian cells. The mammalian interaction was confirmed by cloning the interacting cDNAs in two mammalian expression vectors each containing either the activation domain or the DNA binding domain, respectively. The vectors additionally contained either V5 or His epitopes. HEK293 cells were transfected with the interacting clones and the cells were harvested after 48 hours. The cell lysate was immunoprecipitated with antiV5 or anti-His antibody. The immunoprecipitate was electrophoresed and probed with anti-His or anti-V5 antibody. These interacting clones included SWI/SNF, alpha-tubulin, TAX1 binding protein, and HMG CoA reductase. The involvement of these proteins in tumorigenesis is well documented in the literature. The interaction of RhoGAP with HMG CoA reductase is interesting. It has been reported that statin class of inhibitors of HMG CoA reductase inhibit the growth of breast cancer cells. We postulate that RhoGAP inhibits cell growth by two mechanisms. One, by direct inactivation of Rho proteins. Two, RhoGAP interaction inhibits HMG CoA reductase activity and thus blocks the synthesis of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGP). FPP and GGP are required for modification and translocation of Rho and Ras proteins.

We have analyzed various breast carcinoma cell lines for the expression of the transcripts coding for these interacting proteins. Our data reveals moderate alterations in the levels of these transcripts in breast carcinoma cell lines that point to their involvement in breast tumorigenesis (Figure 6).

Key Research Accomplishments:

- A. We have bacterially expressed the chromosome 13q12 Rho GAP.
- B. The biochemical activity of Rho GAP was characterized.
- C. Mammalian expression constructs of Rho GAP were made and transfected into normal breast breast carcinoma cells and its growth suppression activity demonstrated.
- D. Involvement of p21 in Rho mediated growth suppression was shown.
- E. Rho GAP transfection in NIH3T3 cells led to inhibition of stress fibers.
- F. Yeast two-hybrid system was used to isolate RhoGAP interacting proteins.
- G. Involvement of Rho pathway in statin induced growth inhibition of breast cancer cells was implicated

Reportable Outcomes:

The following manuscript was submitted for consideration of publication.

“Chromosome 13q12 encoded Rho GTPase activating protein suppresses growth of breast carcinoma cells, and yeast two-hybrid screen shows its interaction with several proteins”, by Ganachari M. Nagaraja and Raj P. Kandpal

Conclusions:

We had previously shown that expression of a novel transcript that had sequence similarity to rat Rho GAP sequence was altered in breast carcinoma cell lines. We believed that the putative Rho GAP is functionally active. Rho family of proteins shares homology with the Ras super family. Our working hypothesis was that breast carcinoma where Ras mutations have not been detected could still arise from aberrant Ras signaling by virtue of loss of activity of members of Rho family or factors/proteins that affect the activity of Rho proteins. We have bacterially expressed the putative Rho GAP and shown it to have specificity toward RhoA. To further associate the biochemical activity with its well-established physiological role we have demonstrated that transfected Rho GAP can alter actin organization of fibroblast cell lines. We have transfected breast cancer cells with RhoGAP expression construct and shown it to suppress cell growth. This growth suppression pathway is mediated via p21. To better characterize the physiological significance of RhoGAP we have used the yeast two-hybrid system to isolate interacting proteins. The protein was shown to interact with alpha tubulin, TAX1 binding protein, SWI/SNF and HMG CoA reductase. These interactions provide a mechanism for statin induced inhibition of breast carcinoma cells and indicate as yet uncharacterized involvement of RhoGAP in other processes.

REFERENCES:

1. Hall A. (1990). The cellular functions of small GTP-binding proteins. *Science* 249, 635-640.
2. Aelst, L.V., D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes Dev.* 11, 2295-2322.
3. Hall, A. (1998). Rho GTPases and actin cytoskeleton. *Science* 279, 509-514.
4. Khosravi-Far, R. and Der, C. J. (1994). The Ras signal transduction pathway. *Cancer and Metastasis Reviews* 13, 67-89.
5. Boguski, M.S., and McCormick, F. (1993) Proteins regulating Ras and its relatives, *Nature* 366, 643-654.
6. Coso, O.A., Chiariello, m., Yu, J-C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J.S. (1995) The small GTP binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* 81, 1137-1146.
7. Hill, C.S., Wynne, J., and Treisman, R. (1995) The Rho family GTPases Rho A, Rac1 and Cdc42 regulate transcriptional activation by SRF. *Cell* 81, 1159-1170.
8. Minden, A., Lin, A., Claret, F-X., Abo, A., and Karin, M. (1995) Selective activation of the JNK signaling pathway and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* 81, 1147-1157.
9. Ridley, A.J. (1996) Rho: Theme and variations. *Curr. Biol.* 6, 1256-1264.
10. Olson, M.F., Ashworth, A., and Hall, A. (1995) An essential role for Rho, Rac and Cdc42 GTPases in cell cycle progression through G1. *Science* 269, 1270-1272.
11. Ridley, A.J. (1994). Signal transduction through the GTP-binding proteins Rac and Rho. *J. Cell Sci. Supplement* 18, 127-131.
12. Patterson, H.F., et. al., (1990). Microinjection of recombinant p21 rho induces rapid change in cell morphology. *J. Cell Biol.* 111, 10001.
13. Tominaga, T. et. al., (1993). Inhibition of PMA-induced LFA-1-dependent lymphocyte aggregation by ADP ribosylation of the small GTP binding protein Rho. *J. Cell Biol.* 120, 1529-1537.
14. Takahashi, K. et. al., (1994). Involvement of Rho p21 small GTP-binding protein and its regulation in the HGF-induced cell motility. *Oncogene*, 9, 273-279.
15. Kishi, K. et. al., (1993). Regulation of cytoplasmic division of *Xenopus* embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). *J. Cell. Biol.* 120, 1187-1195.

16. Cerione, R., and Zheng, Y. (1996) The dbl family of oncogenes. *Curr Opin. Cell Biol.* 8, 216-222.
17. Hall, A. (1992). Ras related GTPases and cytoskeleton. *Mol Biol Cell* 3, 475-479.
18. Downward, J. (1992). Rac and Rho in tune. *Nature* 359, 273-274.
19. Kuosravi-Far, R., Solski, P.A., Clark, G.J., Kinch, M.S., and Der, C.J. (1995) Activation of Rac1, RhoA, and mitogen activated protein kinases is required for Ras transformation. *Mol Cell Biol* 15, 6443-6453.
20. Qiu, R., Chen, J., Kirn, D., McCormick, F., Symons, M., (1997) An essential role for Rac in Ras transformation. *Nature* 374, 457-459.
21. Qiu, R., Chen, J., McCormick, F., Symons, M., (1995) A role for Rho in Ras transformation. *Proc Natl Acad Sci USA* 92, 11781-11785.
22. Qiu, R., Chen, Abo, A., McCormick, F., Symons, M., (1997) Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. *Mol Cell Biol* 17, 3449-3458.
23. Keely, P., Parise, L., and Juliano, R. (1998) Integrins and GTPases in tumor cell growth, motility and invasion. *Trend Cell Biol* 8, 101-106.
24. Keely, P., Westwick' J.K., Whitehead, I.P., Der, C.J., and Parise, L.V. (1997) Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI3K. *Nature* 390, 632-636.
25. Talias, K.F., Cantley, L.C., and Carpenter, C.L. (1995) Rho family GTPases bind to phosphoinositide kinases. *J Biol Chem* 270, 17656-17659.
26. Zheng, Y., Bagrodia, S., and Cerione, R.A. (1994) Activation of phosphoinositide-3 kinase activity by cdc42Hs binding to p85. *J Biol Chem* 269, 18727-18730.
27. Manser, E., Leung, T., Salahuddin, H., Zhao, Z., and Lim, L. (1994) A brain serine/threonine protein kinase activated by cdc42 and Rac1. *Nature* 367, 40-46.
28. Burbelo, P.D., Dreschel, D., and Hall, A. (1995) A conserved binding domain defines numerous candidate proteins for both Cdc42 and Rac GTPases. *J Biol Chem* 270, 29071-29074.
29. Settleman, J., Narasimhan, V., Foster, L.C., and Weinberg, R.A. (1992) *Cell*, 69, 539-549.
30. Zohn, I.M., Campbell, S.L., Khosravi-Far, R., Rossman, K.L., and Der, C.J. (1998) Rho family proteins and Ras transformation: The RHOad less traveled gets congested. *Oncogene* 17, 1415-1437.

APPENDIX

Figures 1-6

Tables 1-2

Table 1: GTPase Activation by Rho GAP

Time of Incubation	% Radioactive GTP remaining bound to Rho [@]
0 min	100
5 min	70 \pm 7
10 min	55 \pm 10
30 min	20 \pm 9

[@] Approximately 50 ng of labeled GTP loaded Rho was used to evaluate its GTPase activity. The results are an average of three replicates.

Effect of Recombinant Rho GAP on Rho GTPase activity:

Time of Incubation	% Radioactive GTP remaining bound to Rho ^{@@}
0 min	100
5 min	45 \pm 12
10 min	10 \pm 8

^{@@} The labeled GTP loaded Rho (~50 ng) was mixed with 200 ng of recombinant Rho GAP. The GTPase stimulatory activity of GAP was determined by measuring the amount of labeled GTP remaining bound to Rho.

Table 2: The identity of various cDNAs isolated by using the yeast two-hybrid system.

Gene	Accession Number	Chromosome Location	Biological Function
SWI/SNF, SMARCD3	NM_003078	7q35-q36	Regulation of chromatin remodeling.
Tax1 binding protein (TAX1BP1)	NM_006024	7p15	Anti-apoptosis.
Tubulin alpha 1	NM_006082	12	Member of the tubulin family, involved in polymer formation.
HMG CoA reductase	NM_000859	5q13.3-q14	Rate limiting enzyme in cholesterol biosynthesis.
Novel high mobility group protein (Band 4.1 membrane protein KIAA0338)	AB002336	20	Erythrocyte membrane protein. Its N-terminal interacts with acidic phospholipids in plasma membrane, Glycophorin C and p55, and the C-terminal domain with actin/spectrin.
1,4,5 Inositol Phosphate receptor type I (ITPRI)	NM_002222	3p26-p25	Messenger in signal transduction pathways.
HSP 90 kD protein 1 beta	NM_007355	6p12	Molecular chaperone to facilitate correct protein folding during biogenesis, and translocation in the cell and across membrane.
Brain specific angiogenesis inhibitor 2 (BAI2)	NM_001703	1p35	Angiogenesis
Kinesin superfamily protein KIF1B	AF257176	1p36.2	Microtubule-dependent molecular motors that play important roles in intracellular transport of organelles and in cell division.

RhoGAP	PQS IQQALRYLRSNCLDQVGLFRKSGVKSR IHALRQMNEFPEN
p190	PIFIERCIEYIEATGLSTEGIYRVSGNKSEIESLQRQFDQDHN
ARHGAP9	PSFLRLCIAAVDKRGLDVG IYRVSGNLAVVQKLRLVDRERAVTSDGRV
ARHGAP10	PLIVDICCKLVEERGLEYTG IYRVPGNNAAISSMQEELNKGMAI
p50rho-GAP	PIVLRRETVAYLQAHALTTEGIFRRSANTQVVREVQQKYNMGLP
n-chimaerin	PMVVQMCIREIESRQLNSEGLYRVSGFSDLIEDVKMAFDRLDGEKA
ber	PYIVRQCVEEIERRGMEEVGIYRVSGVATDIQALKAADFVNK
p115	PLVVESCIRFINLNLQHEGIFRVSGAQLRVSEIRDAFERGED

RhoGAP VNYEDQS . . . AYDVADMVKQFFRDLPEPLFTNKLSETF
p190 LDLAEKDFTVNTVAGAMKSFSELPDPLVPYNMQIDL
ARHGAP9	VFPEQPGQEGRLDLSTEDW . DIHVVTGALKLFLRELPPPLVPLLLPHF
ARHGAP10 DIQDDKWR . DLNVISSLLKSFFRKLPEPLFTNDKYADF
p50rho-GAP VDFDQYN . ELHLPAVILKTFLRELPEPLLTDFDLYPHV
n-chimaerin DISVNMYE . DINIITGALKLYFRDLPIPLITYDAYPKF
ber DVSVMSEMDVNAIAGTLKLYFRELPPEPLFTDEFYPNF
p115 PLVEGCTAHDLD SVAGVLKLVFRSLEPPPLFPPDLFGEL

RhoGAP	LHIYQVVSKEQRLQAVQAAILLLADENREVLQTLLCFLNDVNVN LVEENQM
p190	VEAHKINDREQKLHALKEVLKKFPKENHEVFKYVISHLNKVSHNN
ARHGAP9	RAALALSESEQCLSQIQELIGSMPKPNHDTLRYLLEHLRCRVIAHS
ARHGAP10	IEANRKEDPLDRKTLKRLIHDLP EHHYETLKFLSAHLKTVASENKEKNM
p50rho-GAP	VGFLNIDES . QRVPATLQVLQTLPEENYQVLRFLT AFLVQISAHS
n-chimaerin	IESAKIMDPDEQLETLHEALKLLPPAHCETLRYLMAHLKRVTLHE
ber	AEGIALSDPVAKESCMLNLLLSLPEANLLTFLFLDLHLKRVAEKE
p115	LASSELEDTAERVEHVSRLWLRLPAPVLVVLRYLFTFLNHLAQYS

RhoGAP	TPMNLAVCLAPSLFH
p190	
ARHGAP9	
ARHGAP10	EPRNLAIIVFGPTLVRTSEDNMTHMVTHMPDQYKIVETLIQHHD
p50rho-GAP	
n-chimaerin	
ber	
p115	

Figure 1: Alignment of RhoGAP with other proteins.

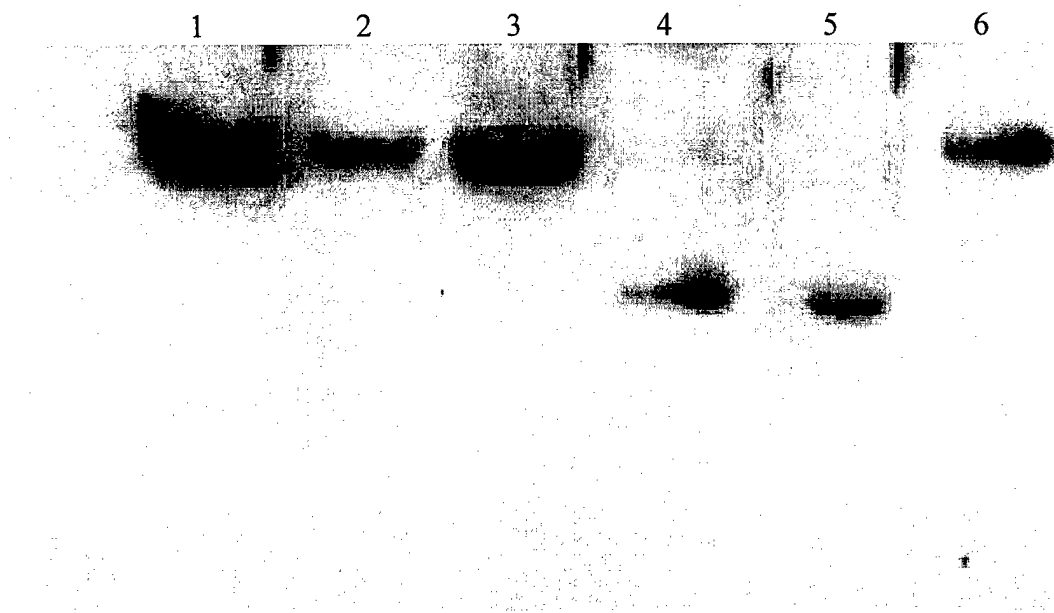


Figure 2: Northern analysis of RhoGAP message. The transcript of ~5.0 Kb RhoGAP message in MCF10A (lane 1), MDAMB431 (lane 2), MDAMB468 (lane 3), and SKBr-3 (lane 6); and a 2.0 Kb message in MCF7 (lane 4) and MDAMB231(lane 5).

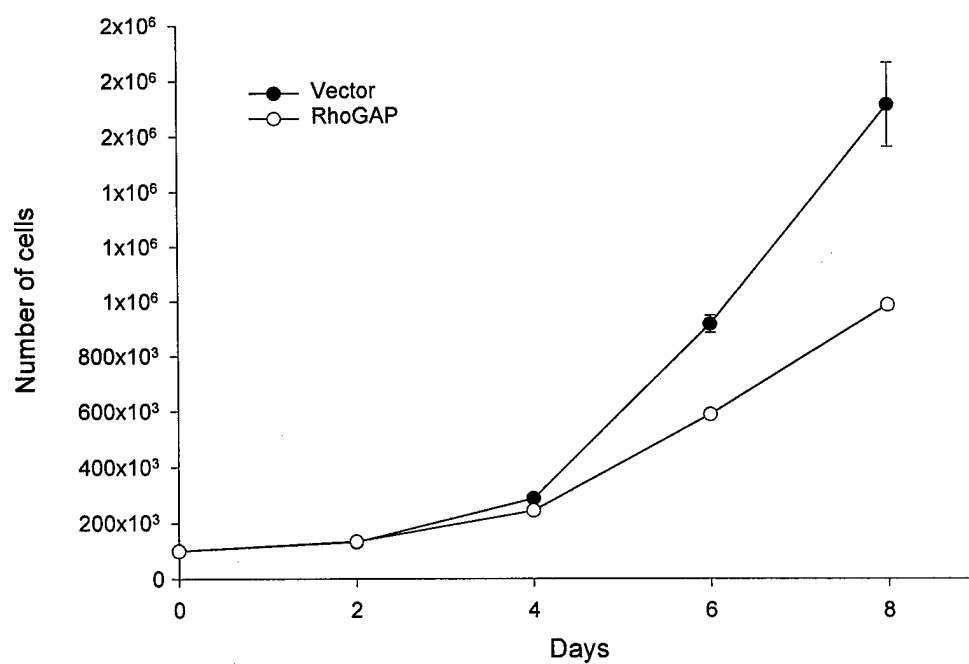


Figure 3: Growth analysis of MCF7 transfected with RhoGAP. Cells on 35 mm culture plate were cultured in medium with 10% FBS. Cell numbers were determined by counting. Results represent mean of 3 or more independent experiments.

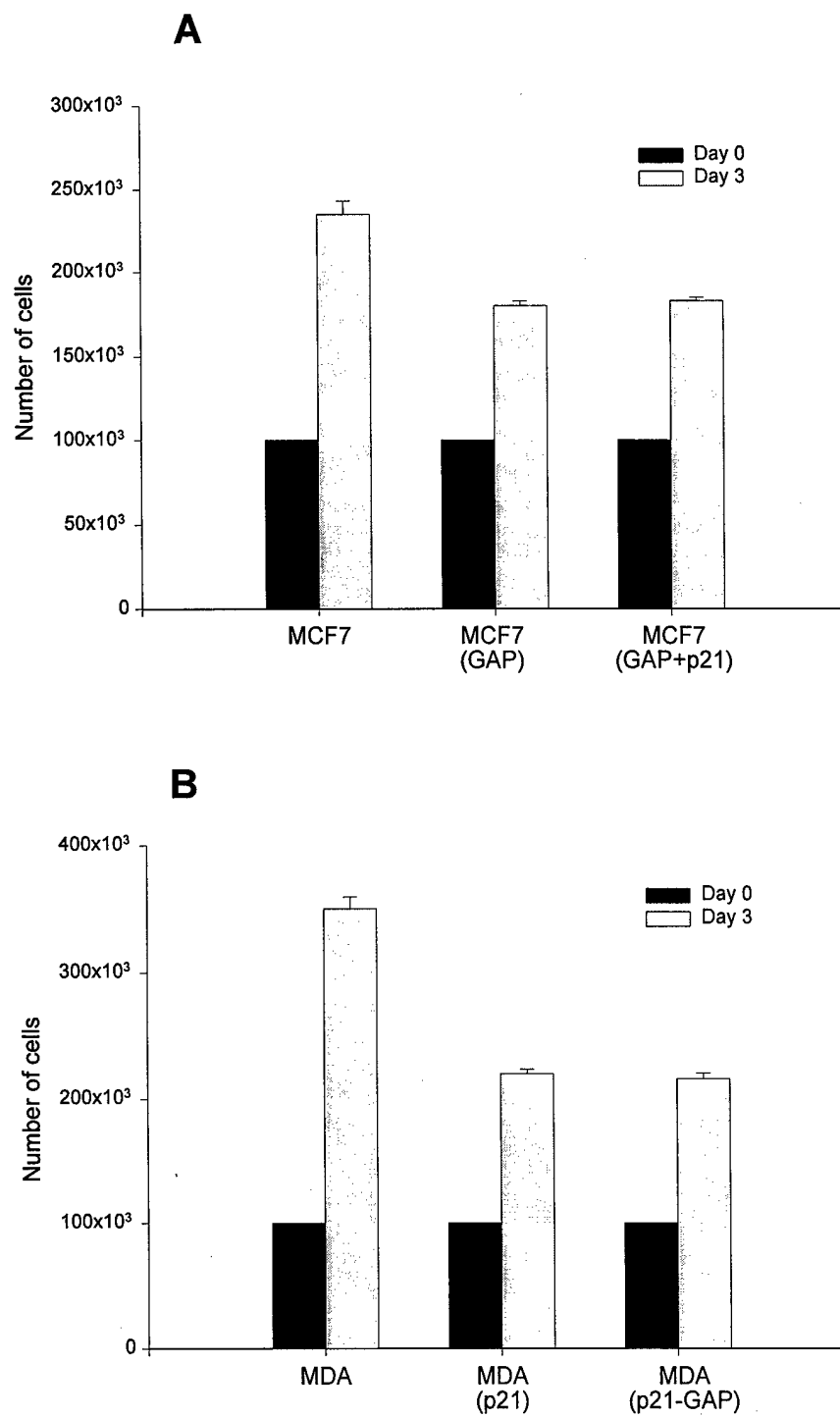


Figure 4: Effect of sequential transfection of RhoGAP + p21 in MCF7 and p21+RhoGAP in MDAMB231 cells on growth.

Table 1: GTPase Activation by Rho GAP

Time of Incubation	% Radioactive GTP remaining bound to Rho [@]
0 min	100
5 min	70 \pm 7
10 min	55 \pm 10
30 min	20 \pm 9

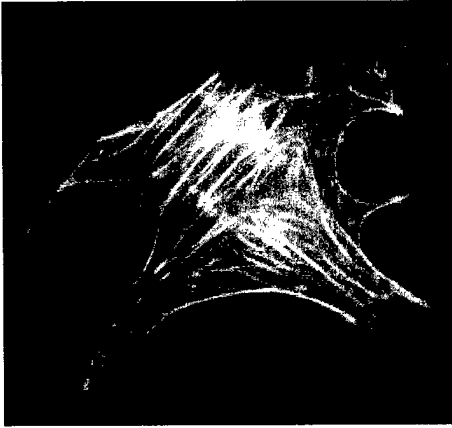
[@] Approximately 50 ng of labeled GTP loaded Rho was used to evaluate its GTPase activity. The results are an average of three replicates.

Effect of Recombinant Rho GAP on Rho GTPase activity:

Time of Incubation	% Radioactive GTP remaining bound to Rho ^{@@}
0 min	100
5 min	45 \pm 12
10 min	10 \pm 8

^{@@} The labeled GTP loaded Rho (~50 ng) was mixed with 200 ng of recombinant Rho GAP. The GTPase stimulatory activity of GAP was determined by measuring the amount of labeled GTP remaining bound to Rho.

(a)



(b)

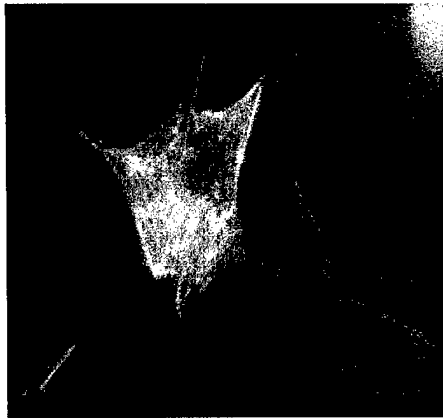


Figure 5: RhoGAP induces the disappearance of stress fibers in NIH3T3 cells. Cells were transfected with vector alone (a) and RhoGAP (b). Cells were fixed and visualized using Texas red Phalloidin.

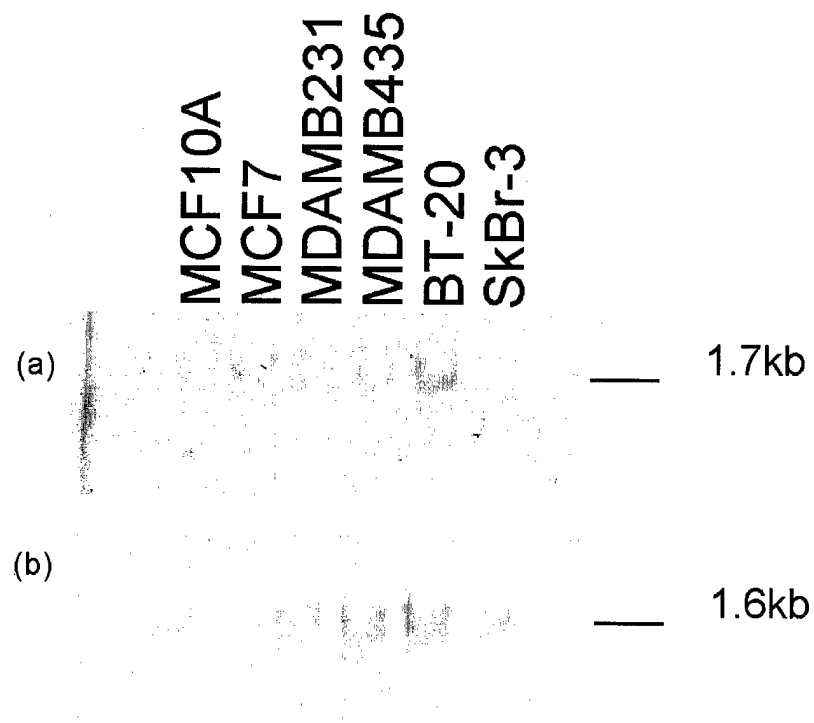


Figure 6: Northern blot analysis of SWI/SNF, (SMARCD3) (a) and Alpha tubulin (b).